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Molecular Cloning of Human GRB-7 Co-amplified with CAB1 and c-ERBB-2 in Primary Gastric Cancer¹

Tatsuya Kishi,*† Hiroki Sasaki,* Nobu Akiyama,* Tomoki Ishizuka,* Hiromi Sakamoto,*
Shigeo Aizawa,† Takashi Sugimura,* and Masaaki Terada*²

*Genetics Division, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104, Japan; and †Second Department of Pathology, Jikei University School of Medicine, 3-25-8, Nishishinbashi, Minato-ku, Tokyo 105, Japan

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Many amplified-chromosome regions in human cancers have been identified, while there are only a limited number of reports for amplified genes in a single amplicon. We recently isolated four cDNA clones, A39, B47, C51, and CAB1, from 500 kilobases of yeast artificial chromosome DNA containing the c-ERBB-2 gene. B47 consisted of 615 base pairs and had about 85% homology with the corresponding sequence of mouse Grb-7. We report here the structure of human GRB-7 cDNA, and we report that the amounts of mRNA for c-ERBB-2, CAB1, and GRB-7 were elevated in concordance with the amplification. © 1997 Academic Press

A variety of proto-oncogene amplification in human tumors has been reported, and there is a high correlation between oncogene amplification and the prognosis of the particular types of tumor (1,2). The overexpression of proto-oncogene through gene amplification is thought to confer a selective growth advantage during tumor progression. The amplicon spreads from hundreds to thousands of kilobases (3-5). We previously reported that *HST1* and *INT2* on chromosome 11q13 were amplified in about 50% of esophageal cancer (6-9). By cosmid walking and exon trapping, we further demonstrated that this amplicon contained at least five genes, *HST1*, *INT2*, MB38, EXP1, cyclin D1/EXP2 genes (10).

Amplification of c-ERBB-2 was reported in about 10% of gastric cancer (11-13). Recently, we successfully obtained four genes, CAB1, B47, A39 and C51, from the 500 kb of yeast artificial chromosome (YAC) DNA containing the c-ERBB-2 gene by the modified cDNA

selection method (Akiyama *et al.*, unpublished observation). The four genes, CAB1, B47, A39 and C51 were co-amplified with c-ERBB-2 in TE 6, esophageal cancer cell line. The nucleotide sequences of A39 and C51 revealed no significant homology to known genes. Predicted amino acid sequence of the CAB1 gene had significant homology to the steroidogenic acute regulatory protein, StAR, which has an essential role in cholesterol transport to mitochondria (14-17). B47 was 615 bp in size, and the nucleotide sequence of B47 had 85% homology to that of the mouse Grb-7 cDNA. Mouse Grb-7 is a SH2 domain protein and was first cloned by the association of the tyrosine phosphorylated carboxyl-terminus of the epidermal growth factor receptor (18), and was found to be a possible signaling adaptor molecule of the receptor-type of tyrosine kinase, c-ERBB-2 product (19). Here we report molecular cloning of human GRB-7, and also show amplification and mRNA expression of five genes, A39, C51, CAB1, GRB-7 and c-ERBB-2, in primary gastric cancers and gastric cancer cell lines.

MATERIALS AND METHODS

Construction of cDNA library and molecular cloning of the human GRB-7. Poly(A)⁺ RNA was prepared from total cellular RNA of TE 6, esophageal cancer cell line, having c-ERBB-2 amplification by oligo(dT)-cellulose column chromatography. The cDNA primed with oligo(dT) was synthesized from 10 µg of the poly(A)⁺ RNA using cDNA Synthesis System Plus kit (Amersham, Arlington Height, IL). The cDNAs of the size more than 0.5 kb were recovered and inserted into λgt10 vector. Two hundred thousand plaques of the constructed λgt10 cDNA library were screened by plaque hybridization using ³²P-labeled mouse Grb-7 cDNA as a probe. The mouse Grb-7 probe was prepared from 1 µg of mouse liver poly(A)⁺ RNA by reverse transcription-PCR. The sequences of mouse Grb-7 primers were: 5'-AAGCTG-TATGGGATGCC-3' and 5'-GCTGCAGCAGGTCTGTGAAAC-3'. Twenty three cDNA clones were obtained. Six out of 23 clones had the size of 2.3 kb corresponding to the size of human GRB-7 mRNA. Five out of six clones could be classified into the same group by restriction enzyme mapping. The insert DNAs of the two candidate full length clones of human GRB-7 were cleaved from the vector by EcoRI-digestion and subcloned into pUC18 vector. DNA sequence

¹ The nucleotide sequence reported in this paper has been deposited in the DDBJ, EMBL, and GenBank databases under Accession No. D43772 for human GRB-7.

² To whom correspondence should be addressed. Fax: +81-3-3248-0326.

was performed by Applied Biosystems 370A DNA sequencer (Applied Biosystems, Foster City, CA).

Cell lines and tumor tissues. The nine gastric carcinoma cell lines used in this study are the following: MKN7, MKN28, MKN74, MKN45, OKAJIMA, TMK-1, KATO-III, HSC-39 and MKN1 (20). These cell lines were maintained in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum at 37°C under a humidified atmosphere of 5% CO₂. A total of 70 primary gastric carcinoma tissues and their adjacent normal tissues were surgically removed at the National Cancer Center Hospital, Tokyo.

DNA preparation and Southern blot analysis. Genomic DNA was prepared from cell lines and tissues using a standard method. The extracted DNA was digested with EcoRI, and 10 µg of digested DNA was fractionated on 0.8% agarose gel and transferred to Hybond-N+ (Amersham). Hybridization was carried out in 50% formamide, 5× standard saline citrate (SSC) (1× standard saline citrate = 0.15M NaCl, 0.015M sodium citrate (SDS)), 5× Denhardt's solution, 5mM EDTA, 0.1% sodium dodecyl sulfate, 10% dextran sulfate, and 100 µg/ml denatured salmon sperm DNA at 42°C for 14-16h. All DNA probes were labeled with α-[³²P]dCTP using a rediprime DNA labelling system kit (Amersham). The filter was washed twice in 0.1× SSC and 0.1% SDS at room temperature for 10min each and then washed at 65°C for 30min, and exposed to Kodak XAR film at -70°C. The hybridization intensity in each sample was quantified by Bio-image Analyzer (BAS2000; Fujix, Kanagawa, Japan). As a control for the equal loading, transfer and hybridization of the DNA samples, the filters were stripped and re-hybridized with probes for HOX2I gene which is located on the same chromosome 17 (21). The intensities of the HOX2I hybridizing fragments were used as references for normalization of the results. The criterion for gene amplification used in this study was that the intensity of the hybridization signal was elevated more than 3-fold compared with the normal one.

RNA preparation and northern blot analysis. Poly(A)⁺ RNA was prepared from culture cells using Fast track kit (Invitrogen, San Diego, CA), and total cellular RNA was prepared from surgical tissues using ISOGEN kit (Nippon Gene, Toyama, Japan) according to the recommended procedure by the supplier. Two µg of poly(A)⁺ RNA or 20 µg of total cellular RNA was electrophoresed on 1% agarose-formaldehyde gel and transferred to NitroPlus membrane (Micron Separations, Inc., Westboro, MA). The filter was hybridized with radiolabeled probes under the same conditions as described above.

Probes. The following probes were used in Southern and northern blot hybridization. The c-ERBB-2 probe was prepared from pCERNH7 constructed from pCER235 and pCER204, a full length cDNA clone (22). The CAB1 probe was a 2.0 kb fragment of the full length cDNA clone. The B47/GRB-7, A39 and C51 probes were cDNA fragments previously cloned from c-ERBB-2 locus by the cDNA enrichment method (Akiyama *et al.*, under submission). The B47/GRB-7 probe was 615 nucleotides (corresponding to nucleotide 1072-1686), A39 was 600 nucleotides and C51 was 670 nucleotides. The HOX2I probe consisted of a PCR product encompassing nucleotides 20-561 (21). The rat β-actin cDNA originally cloned was used as an internal control probe in northern blot hybridization.

RESULTS

The oligo(dT)-primed cDNA library inserted into λgt10 vector was constructed from the RNA of TE 6 cells, and screened by a mouse Grb-7 cDNA fragment corresponding to nucleotides 1323-1975 (18). One clone of about 2.3 kb was entirely sequenced by Applied Biosystems 370A DNA sequencer. The sequence of this cDNA represents an open reading frame of 1596 nucleotides encoding 532 amino acids containing nucleotides

GCCCCCGGATCCACAGGGCTCCCCCGGCCCTGACTCTCTGTCGAAGTCGGGACAC	60
CCTCTTACCAACCTGTAGAGAGCGGGAGTGATCTGAAATAAAAATCCAGAACTGGGGG	120
TCTCTAGACGGAGGCCAACACTCGGAAGCGGTGTCCTGACTCCCTGCCTGGGGCTCC	180
GACAAAGGGCACACAACACTGGTCCGTAAGCCCCTCTCGCTCAGACGGCATGGACTG	240
M E L 3	
GATCTGTCACCATCTCATCTAGCACCTCTCGGAAGACCTTTGGCCAGCCCCCTGGACC	300
D L S P P H L S S S P E D L W P A P G T	23
CCTCTCTGGACTCCCGGCCCTGTAGACCCCTCTGCCCTGAGGGAGTAAAGAGGTCCCAG	360
P P G T P R P D T P L P E E V K R S Q	43
CCTCTCTCATCCAAACCCACCGCAGGAAACTTCGAGAGGGAGGAGGGCTGCCACCTCC	420
P L L I P T G R K L R E E R R A T S	63
CTCCCCCTCATCCCAAACCCCTCTCTGAGCTCTGCACTCCTCACAGGCCATT	480
L P S I P N P F F P E L C S P P S O S P I	83
CTCGGGGGCCCCCTCAGTGCAGGGGCTGCTCCCGGATGCCAGCCCOCCCATGTA	540
L G G P S A R G S L P R D A S R P H V	103
GTAAAGGGTACAGTGGAGATGGGGCTGCAGGCTGTGGAGGTGGAGGAGGTGGCCACA	600
V K V Y S E D G A C R S V E V A A G A T	660
GCTCGGACCGTGTGTAATGCTGTGTCAGCGAGCTCACGCCCTTGAGGCCAGGACCTGG	123
A R H V C E N L V O R A H A L S D E T N	143
GGGCTGGTGGAGTGGCCACCCCAACTGCACTGGAGCCGGGTTTGAGGACACAGATGC	1720
G L V E C C P H I L A L E R G L E D H E S	163
GTGGTGGAACTGCAAGGCTGGCCCTGGCCGGAGATAGCCCTTCGCTTCCGGAAA	780
V V E V O A A M P V G G D S R F V F R K	183
AACCTCCCAAGTCAAGCAACTGTCAGACGCTCCCAACTCCCTGTCCAGAAGAAAATG	840
N F A K Y E L F K S S P H S L F P E K M	203
CTCTCAGGCTGTCTGGATGCACACACTGTTATATCCCATGAAAGACCTCATCCAGAACCTC	900
V S S C L D A H T G I S H E D L I Q N F	223
CTGAGATGCTGGCAGCTTCTGAGATCTGGAGCTTCTGAGCTGGGGGTTCAAGGACCG	960
L N A G S F P E I D O F L R L G S G R	243
AAGCTTGGAAAGCTTCTGAGATCTGGCTTCTGGCCGATCTGGCTTATTAACCTCACCAAG	1020
R L W K R F F C F L R R S G L Y Y S T K	263
GGCACCTCTAAGGATCCGAGGACCTCGAGTACCTGGAGATGTGAAAGGAGTCACCGT	1080
G T S K D P R H L O Y V A D V N E S N V	283
TACGTGGTAGCCAGGCGAACGCTTACGGATGCCACTGACTTCGGTTCTGTGTC	1140
Y V T Q G R K L Y G M P T D F G F C V	303
AAGCCCAACAAAGCTTCTGAAATGGGACACAAGGGGCTTCGAGCTTCTGAGTAAGATGAG	1200
K P N K L R N G H K G L I F C S E D E	323
CAAGCGCGCACCTGCTGGCTGGCTCCCTCCGCTTCTGAGTACGGCTGCCACCTGTAC	1260
Q S R T C W L A A F R L F K Y G V Q L Y	343
AAGAATTCCAGGACAGCACAGTCTGGCCATCTGAGATCCATCTGTTGGCTCCCCACCC	1320
K N Y Q O A S R H L P S C L G S P P	363
TTGAGAACTGCTCTGAGATAATACCCCTGGCCATGAGCTCTCTGGCCATGCTGGCGT	1380
L R S A S D N T V A M D F S G H A G R	383
GTCATTGAGAACCCCCGGAGGCTCTGAGTGTGGCTTGGAGAGGGCCAGGGCTGGAGG	1440
V I E N P R E A L S V A L E E A O A W R	403
AAGAGACACACCCACCGCCCTAGCGCTGCCATGCGACCTCCCGCACGAGCTCAGTGA	1500
K K D R L S P L M P A S G T S L S A	423
GCCATCCACCGCAGCCAACTCTGGCTCCACGGGGCATTTCCGGTGGAGGAGGCCACGG	1560
A I H R T O L W F E R G S R E E E S Q R	443
CTTATTGGACAGCAGGGCTTGAGACGGCTGTCTGGCTGGAGACTCAGGGAAAC	1620
GGCGGGCTTGTCTCTGAGATCTGGCTGGCTGGCTGGCTGGCTGGCTGGCTGGCT	463
CCCCAGGGCTTGTCTCTTGTGCAACCTCGAGAAAGTGAAGCATTATCTCATCTC	1680
CGCGAGGGATGG	483
CGGAGGGAGGG	1740
TCCTCTAGATAGAAAACAGCCCCCACTCCAGTCAGCTCCACCTCCACCCCTCTCTCA	503
GCCCTTGCGGTGGCCCCCTCTCCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCA	1800
TTATGAGAGTGG	523
CGCCATTCGCTGCCAGCGGGGGCCCTGAGCTGAGCCGGCTGGCTCATGCCCTAGCC	1860
CGCCCTTCAAGCTGCCAGCGGGGGCCCTGAGCTGAGCCGGCTGGCTCATGCCCTAGCC	532
GGGGCGGG	1920
TCCTCTAGATAGAAAACAGCCCCCACTCCAGTCAGCTCCACCTCCACCCCTCTCTCA	1980
GCCCTTGCGGTGGCCCCCTCTCCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCA	2040
TTATGAGAGTGG	2100
AGCCAGGTGATCTGCTCTGTTTATAGTAGTGACAAATAAGATTATTTTGATACAAA	2160
AAAAAAAAAAAAAA	2220
	2235

FIG. 1. Nucleotide and predicted amino acid sequences of human GRB-7 cDNA. Nucleotide and amino acid positions are indicated to the right of the sequence. The shaded region corresponds to the SH2 domain.

sequence of the B47 clone (Fig. 1). The first in-frame ATG codon was at nucleotide position 220 and was preceded by an in-frame terminator codon TAA 132 nucleotides upstream. The nucleotides sequence flanking the start methionine conforms to the Kozak consensus sequence for initiation of protein synthesis (23). Deduced amino acid sequence of human GRB-7 revealed 90% identity to that of mouse Grb-7 (Fig. 2). Human GRB-7 has one SH2 domain at its carboxyl terminus corresponding to amino acids 432-532, and the amino-terminal sequence is proline-rich. The central portion of

mGRB7	MELDLSPTHLSSSPEDVCPTATPPETPPPPDNPPPGDVKRSQPLPIP-SSRKLREEEFQ	59
hGRB7	MELDLSPPHLSSSPEDLWPAFGTPPGTPRPPDTLPPEEVKRSQPLLIPTTGRKLREERR	60
mGRB7	ATSLPSIPNPFPPELCSPPSQKPILGGSSGARGLLPRDSSRLCVVKVYSEDGACRSVEAA	119
hGRB7	ATSLPSIPNPFPPELCSPPSQSPILGGCPSSARGLLPRDASRPHVVKVYSEDGACRSVEAA	120
mGRB7	GATARHVCEMI.VQRHALSDESWGLVESHPYLALERGLIEDHEFVVVEQEAWPVGGSRFI	179
hGRB7	GATARHVCEMI.VQRHALSDETGWGLVECHPKHLALERGLEDHESVVEVQAAWPVGGSRFV	180
mGRB7	FRIONFAKYELFKSPPHPTLFPEKVMSSCLDAQTGISHEDLQNFLNAGSFPEIQQGFLQLRG	239
hGRB7	FRIONFAKYELFKSPPHSLSFPEKVMSSCLDAHTGISHEDLQNFLNAGSFPEIQQGFLQL--	238
mGRB7	SGRGSGRKWLWKRFFCFLRRSGLYYSTKGTSKDPRHLQYVADVNESENVYVVTQGRKLYGMP	299
hGRB7	--RGSGRKWLWKRFFCFLRRSGLYYSTKGTSKDPRHLQYVADVNESENVYVVTQGRKLYGMP	296
mGRB7	TDFGFcvkPNKLRLNGHKGLHIFCSDEQSRTCWLAFLRFKYGVLQYKNYQQAQSRHLR	359
hGRB7	TDFGFcvkPNKLRLNGHKGLRIFCSDEQSRTCWLAFLRFKYGVLQYKNYQQAQSRHLHP	356
mGRB7	SYLGSPPLRSVSDNTIVAMDFSGHAGCRVIDNPREALSAAMEEAQAWRKTKTNHRLSLPTTC	419
hGRB7	SCLGSPPPLRSASDNTIVAMDFSGHAGCRVIDNPREALSALEEQAQAWRKTKTNHRLSLPMPA	416
mGRB7	SGSSLSSAIIHRTQPWFHGRISREESQRLLGQQCLVDGVFLVRESQRNPQGFVLSICLHQK	479
hGRB7	SGTSLSAAIIHRTQNLNFHGRISREESQRLLGQQGLVDCGLELVRESQRNPQGFVLSICLHQK	476
mGRB7	VKHYLILPSEDEGGLYFSMDEGQTRFTDILQLQVLFHQINRGLPCLLRHCCARVAL	535
hGRB7	VKHYLILPSEEEGRLYFSMDGGQTRFTDILQLQVLFHQINRGLPCLLRHCCTRVAL	532

FIG. 2. Amino acid comparison between mouse *Grb-7* (mGRB7) and human *GRB-7* (hGRB7). Identical amino acids are indicated by vertical dashes. The boxed region is a SH2 domain and the underlined region is a plekstrin domain. The repeated four amino acids (Arg-Gly-Ser-Gly) corresponding to 238-241 of mouse *Grb-7* were deleted in human *GRB-7*.

mouse *Grb-7* contains a plekstrin domain (24-26). The plekstrin domain of human *GRB-7* corresponds to amino acids 231-336. The amino acid sequences of the SH2 and plekstrin domains are almost conserved between human and mouse. The repeated motif which consists of four amino acids Arg-Gly-Ser-Gly in the plekstrin domain, is deleted in human *GRB-7*.

The amplification of *c-ERBB-2*, *CAB1*, *GRB-7*, A39 and C51 genes was investigated in primary gastric cancers and gastric cancer cell lines. The DNA samples extracted from gastric cancer tissues were digested with *Eco*RI and analyzed by Southern blot hybridization. Representative results of Southern blot analyses are shown in Fig. 3. The presence of equal amounts of DNA in each lane was confirmed by the hybridization with the probe of *HOX2I* gene, which was located on the same chromosome 17 (Fig. 3). In these samples, co-amplification with *c-ERBB-2* was found in samples by hybridization with the *GRB-7*, C51 and *CAB1*, in no sample with the A39 probe, respectively. Nine gastric cancer cell lines were analyzed for the amplification of the five genes. Only the MKN7 gastric cancer cell line showed amplification of *c-ERBB-2*, *CAB1*, *GRB-7*, A39 and C51 genes (data not shown).

The total cellular RNAs prepared from three pairs of tumor tissue samples (T15, T16, T17) and the corresponding adjacent normal tissue samples (N15, N16,

N17) were tested by northern blot hybridization with *c-ERBB-2*, *CAB1*, *GRB-7*, A39 and C51 probes. All the tumor samples contained a significantly high amount of *c-ERBB-2*, *CAB1* and *GRB-7* mRNA, while the corresponding normal tissues contained little (or none) (Fig. 4.). More than 8-fold amplification of *c-ERBB-2* and *GRB-7* was observed in the T16 DNA sample, while *CAB1* was amplified 3-fold. With correspondence to the amplification degree of these genes, the T16 RNA sample contained markedly increased amounts of mRNAs for *c-ERBB-2* and *GRB-7*, but the amount of *CAB1* was not remarkably increased. mRNAs for A39 and C51 were not detectable in all three pairs of samples. The poly(A)⁺ RNAs prepared from the nine gastric cancer cell lines were analyzed by northern blot analysis for expression of these five genes. All the gastric cancer cell lines contained mRNAs for *c-ERBB-2*, *CAB1* and *GRB-7*. Markedly elevated amounts of *c-ERBB-2*, *CAB1* and *GRB-7* mRNA were found in MKN7 cells compared to the amounts in other cell lines. The mRNAs for A39 and C51 were not detected in all the cell lines.

DISCUSSION

Gene amplification of human tumors has been examined as a proto-oncogene amplification. There have

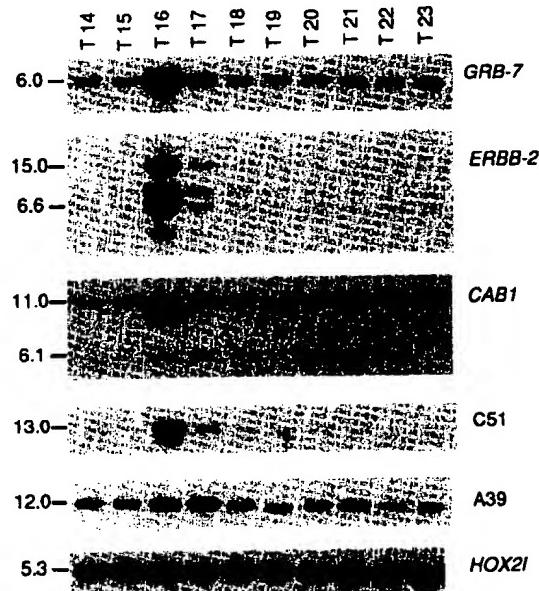


FIG. 3. Southern blot analysis of DNA from gastric cancer tissues. The case number is given on top. *Eco*-RI digested DNA was hybridized with *GRB-7*, *c-ERBB-2*, *CAB1*, C51, A39, and *HOX2I* probes. Probes are shown at the right side. The approximate sizes (in kilobases) of the represented bands are shown at the left side. As control for the loading amount of DNA the blots were hybridized with the *HOX2I* probe.

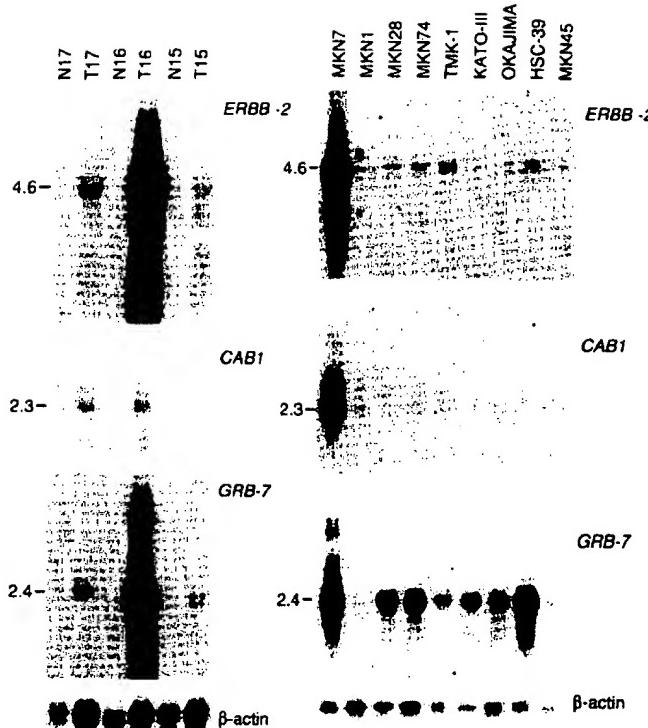


FIG. 4. Northern blot analysis of the *c-ERBB-2*, *CAB1* and *GRB-7* genes in the gastric cancer tissues and gastric cancer cell lines. (Left) Twenty μ g of total cellular RNA from three pairs of gastric cancer tissues (T15, T16 and T17) and matched adjacent normal tissues (N15, N16 and N17) were subjected to blot hybridization. (Right) Two μ g of poly(A)⁺RNA from the nine gastric cancer cell lines were subjected to blot hybridization. Probes are shown at left side. Approximate sizes (in kilobases) of the major transcripts are shown at right side. As an internal control, β -actin probe was hybridized.

been, however, only limited reports available which analyze the genes located in the amplified region. The investigation of genes on the amplicon will lead to an understanding of genes giving selective growth advantage to cells. It was reported that there was a DEAD box gene, *HuDBP-RB/DDX1*, on *N-MYC* amplicon (27), and the 12q13-14 locus amplified in human sarcoma or glioma contained six genes including *CDK4*, *SAS* and *MDM2* (28-30). In this study, *c-ERBB-2* amplification was found in the primary gastric cancers, all originated from differentiated types in agreement with previous reports (11). The other four genes, *CAB1*, *GRB-7*, A39 and C51, were amplified only in the samples in which *c-ERBB-2* was also amplified. The present data together with the results on the analysis of 11q13 amplicon, *N-MYC* amplicon and 12q13-14 amplicon established that there are multiple-expressed genes in a single amplicon.

The *GRB-7* gene encodes a SH2 domain protein and a sequence similar to the pleckstrin domain which is supposed to mediate protein-protein interaction during

signal transduction (25,26). The amino terminal portion of *GRB-7* is proline rich and may be a potential binding site for SH3 domain. The product of mouse *Grb-7* binds tightly to *c-ERBB-2* through its SH2 domain in the breast cancer cell lines, and co-amplification of *GRB-7* and *c-ERBB-2* in human breast cancer cell lines has been reported (19). Although it has not been clarified what signal might be sent through *GRB-7*, it is conceivable that the simultaneous overexpression of a receptor tyrosine kinase and a coupling SH2 domain protein may enhance the signal transduction pathway and lead cells to proliferation. Our results suggest that in primary gastric cancers with *c-ERBB-2* amplification, co-amplification of *GRB-7* and *c-ERBB-2* may lead to a more aggressive phenotype. The *c-ERBB-2* gene had been analyzed as a prognostic marker in cancer patients, especially in breast cancer (31, 32). The analysis of *GRB-7* amplification in addition to *c-ERBB-2* amplification may underscore the role of *c-ERBB-2* as a prognostic marker.

It has been noted that *c-ERBB-2* and *THRA-1*/thyroid hormone receptor alpha gene, the cellular homologue of the viral *erb-A* oncogene, were co-amplified in breast and gastric cancer (11, 33). The amount of *THRA-1* mRNA was not elevated in spite of amplification. In contrast, mRNA expression of *GRB7*, *c-ERBB-2* and *CAB1* was elevated in concordance with amplification. No mRNA for C51 and A39 was detected by northern blot hybridization despite amplification. In general, the key gene providing the selective force for tumor progression should fulfill two criteria: the first is that it should be affected in all cases of the amplification, and the second is that it should be expressed at elevated levels in the tumors. Therefore, *GRB7* and *CAB1*, as well as *c-ERBB-2*, seem to be candidate genes for a leading role by this amplicon in gastric cancer and probably also in breast cancer and ovarian cancer.

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